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Prejunctional muscarinic receptors from the deep muscular plexus of canine ileum were studied, and their properties compared to the postjunctional receptors of the circular smooth muscle. In the purified synaptosomal fraction (a fraction containing primarily the axonal varicosities of deep muscular plexus), the muscarinic ligand [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) labelled an apparently homogenous population of receptors (n_H = 1) with a K_d of 2.7 nM and a B_{max} of 195 ± 44 fmol/mg protein (mean ± S.D., n=4). These receptors showed a high affinity for the M_3/M_1-selective antagonist 4-DAMP (pK_i = 7.41); in contrast, the pK_i values of pirenzepine (5.60), methoctramine (5.65), and AF-DX 116 (5.21) implied little selectivity for these subtypes. The binding properties of muscarinic receptors in the synaptosomal fraction were different from the binding properties of muscarinic receptors in the purified circular smooth muscle plasma membranes. Most notably, the circular smooth muscle receptors had significantly lower affinity for [^{3}H]-NMS (K_d = 16 nM) with a B_{max} value of 2088 ± 276 fmol/mg. The affinities of the M_2 subtype selective muscarinic antagonists methoctramine and AF-DX 116 were similar in both membrane preparations. The receptor population associated with the deep muscular plexus synaptosomal fraction was linked to the inhibition of adenylate cyclase activity, as demonstrated by a concentration-dependent, atropine-sensitive inhibition of the forskolin-stimulated enzyme in the presence of muscarinic agonists carbachol and oxotremorine. Based on the pharmacological observations presented here, the prejunctional muscarinic receptors in the axonal varicosities of deep muscular plexus are different from the postjunctional receptors present in the circular smooth muscle.

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Prejunctional Muscarinic Receptors in the Deep Muscular Plexus of Canine Ileum: Comparison with Smooth Muscle Receptors[1]

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ABSTRACT

Prejunctional muscarinic receptors from the deep muscular plexus of canine ileum were studied, and their properties were compared with those of the postjunctional receptors of the circular smooth muscle. In the purified synaptosomal fraction (a fraction containing primarily the axonal varicosities of deep muscular plexus), the muscarinic ligand N-[^{3}H]methylscopolamine labeled an apparently homogenous population of receptors (n_H = 1) with a K_d of 2.7 nM and a B_{max} of 195 ± 44 fmol/mg protein (mean ± S.D., n = 4). These receptors showed a high affinity for the M_3/M_1-selective antagonist 4-diphenylacetoxy-N-methylpiperidine methiodide (pK_i = 7.41); in contrast, the pK_i values of pirenzepine (5.60), methoctramine (5.65) and AF-DX 116 (5.21) implied little selectivity for these subtypes. The binding properties of muscarinic receptors in the synaptosomal fraction were different from the binding properties of muscarinic receptors in the purified circular smooth muscle plasma membranes. Most notably, the circular smooth muscle receptors had significantly lower affinity for N-[^{3}H]methylscopolamine (K_d = 16 nM) with a B_{max} value of 2088 ± 276 fmol/mg. The affinities of the M_2 subtype-selective muscarinic antagonists methoctramine and AF-DX 116 were similar in both membrane preparations. The receptor population associated with the deep muscular plexus synaptosomal fraction was linked to the inhibition of adenylate cyclase activity, as demonstrated by a concentration-dependent, atropine-sensitive inhibition of the forskolin-stimulated enzyme in the presence of muscarinic agonists carbachol and oxotremorine. Based on the pharmacological observations presented here, the prejunctional muscarinic receptors in the axonal varicosities of deep muscular plexus are different from the postjunctional receptors present in the circular smooth muscle.

Most investigations of the gastrointestinal tract have focused on postjunctional receptors associated with various gastrointestinal smooth muscles (Giraldo *et al.*, 1987, 1988; Muommi *et al.*, 1988; Herawi *et al.*, 1988; Lucchesi *et al.*, 1989). These receptors mediate the direct excitatory effects of acetylcholine on the contractile activity of smooth muscle cells. The muscarinic receptor-mediated effects of acetylcholine on the contractile responses in the gastrointestinal tract also involve an indirect component resulting from the activation of receptors present in the enteric nervous system.

In contrast to the postjunctional receptors, the pharmacological properties of neural muscarinic receptors are less clearly defined. In the ileum, muscarinic receptors have been identified in ganglionic plexuses (myenteric and submucosal) of guinea pig and dog. The pharmacological properties of these receptors have been elucidated primarily by functional studies or by monitoring the activities of single neurons by electrophysiological techniques (Kilbinger, 1984; Fox *et al.*, 1985; North *et al.*, 1985; Schworer and Kilbinger, 1988; Schiavone *et al.*, 1989). It has been shown that muscarinic receptors in the myenteric and submucosal plexuses consist of pharmacologically heterogenous populations, mediating both excitatory and inhibitory responses. It has been suggested that the excitatory receptors, having a high affinity for pirenzepine, are located postsynaptically on neuronal somas or dendrites. The inhibitory receptors, exhibiting a low affinity for pirenzepine, were associated with the axonal varicosities of myenteric and submucosal nerves, mediating the prejunctional inhibition of neurotransmitter release (Kilbinger and Nafziger, 1985; North *et al.*, 1985; Hashimoto *et al.*, 1986).

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[2] This work was completed while P.K. held a National Research Council-Walter Reed Army Institute of Research Fellowship.
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ABBREVIATIONS: [^{3}H]NMS, N-[^{3}H]methylscopolamine; 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine methiodide; MOPS, 3-(N-morpholino)propanesulfonic acid; cAMP, cyclic AMP.

Recently, it was shown that the binding properties of prejunctional muscarinic receptors in the myenteric plexus of canine ileum can be discriminated from the binding properties of smooth muscle receptors if the binding assay were performed with highly purified membrane materials (Kostka *et al.*, 1989a). The present study extends these initial observations by examining the pharmacological properties of prejunctional receptors in the deep muscular plexus. This plexus, embedded within the circular muscle layer, is formed by a network of neurites extending from both myenteric and submucosal plexuses (Furness and Costa, 1987; Herman and Bass, 1990). Thus, deep muscular plexus should be suitable for the characterization of receptors associated with axonal varicosities because 1) the deep muscular plexus lacks neuronal cell bodies and 2) the interferences of receptors derived from neuronal somas will not contribute to the binding properties of prejunctional receptors.

Materials and Methods

Materials. [^{3}H]NMS (specific activity 85–87 Ci/mmol) and [α-^{32}P]ATP (approximately 2000 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Methoctramine and 4-DAMP were obtained from Research Biochemicals Inc. (Natick, NA). Atropine was a product of Sigma Chemical Co. (St. Louis, MO). Pirenzepine and AF-DX 116 were provided by Karl Thomæ GmBH (Biberach, Germany). All other analytical grade chemicals were purchased from commercial sources.

Membrane preparation. The subcellular fractionation of circular smooth muscle/deep muscular plexus was performed as previously described (Ahmad *et al.*, 1987, 1988). Briefly, the circular smooth muscle/deep muscular plexus was separated from the longitudinal muscle layer and submucosa by mechanical dissection. Routinely, membrane preparations were obtained from 15 to 20 g of circular smooth muscle dissected from the whole length of dog ileum. By light microscopic examination, the circular smooth muscle strips were devoid of submucosa, and contaminating submucosa and longitudinal muscle was estimated to not exceed 1% to 2% of the tissue muscle. The tissue was suspended in the isolation buffer (250 mM sucrose, 10 mM $MgCl_2$ and 20 mM MOPS-NaOH pH 7.4) at wet weight/volume ratio of 1:10, and then the tissue was minced and homogenized for 20 sec (Polytron PT 20, 15,000 rpm). The homogenate was centrifuged at 1,000 × *g* for 10 min, and the supernatant was next centrifuged at 10,000 × *g* for 10 min. The pellet (crude synaptosomal fraction) was saved, and the supernatant was used to prepare the crude microsomal pellet by centrifugation at 170,000 × *g* for 60 min. The pellet was resuspended in the isolation buffer and centrifuged at 10,000 × *g* for 10 min to yield the refined microsomal fraction as a supernatant. 1) The purified synaptosomal fraction was prepared by sucrose density gradient fractionation of the crude synaptosomal fraction. The gradients consisted of 2 ml each of 14%, 25%, 35%, 40% and 48% sucrose solutions. After the centrifugation at 110,000 × *g* for 100 min, the purified synaptosomes were obtained by combining the protein bands at the 35%/40% and 40%/48% sucrose interfaces. 2) The purified smooth muscle plasma membranes of circular smooth muscle were obtained by fractionation of the refined microsomal fraction on sucrose density gradients (110,000 × *g* × 100 min) consisting of 2.5 ml each of 14%, 33%, 40% and 48% sucrose. The purified fraction of smooth muscle plasmalemma was recovered from the 14%/33% sucrose interface.

The relative enrichment of axonal varicosities in the purified synaptosomal fraction and of smooth muscle plasmalemma in the fraction of smooth muscle plasma membranes was based on the activities of tetrodotoxin-sensitive [^{3}H]saxitoxin binding, a marker of neuronal plasma membranes, and of 5′-AMPase, a marker of smooth muscle plasma membranes (Ahmad *et al.*, 1988; Kostka *et al.*, 1987). The purified synaptosomal fraction was enriched approximately 20-fold in the [^{3}H]saxitoxin binding over the postnuclear supernatant, and the preparation of smooth muscle plasma membranes was approximately 20 fold enriched in 5′-AMPase activity (Ahmad *et al.*, 1987, 1988).

Binding assay. The assay of [^{3}H]NMS binding was carried out as described previously (Kostka *et al.*, 1989a). Briefly, the membrane fractions were incubated with the ligand for 20 min at 37 C in a media buffered to pH 7.4 by 50 mM MOPS-imidazole. The incubation was terminated by dilution of samples with ice-cold buffer (10 mM MOPS-imidazole, 0.25 M sucrose, pH 7.4) followed by rapid filtration under suction using Whatman GF/F filters. The specific binding was determined as a portion of total binding inhibited by 10 μM atropine. The atropine-insensitive retention of ligand represented less than 15% of the total binding.

The competition studies were conducted simultaneously on both synaptosomal and smooth muscle plasma membrane-enriched fractions prepared from the same starting material. Typically, the [^{3}H]NMS concentrations in the competition experiments were near or above the K_d value for a particular receptor population (3–5 nM for synaptosomal fraction; 15–20 nM for smooth muscle plasma membranes).

Adenylate cyclase. The adenylate cyclase activity in the crude synaptosomal fraction was determined as previously described (Kostka *et al.*, 1989c). The reaction mixtures contained 75 mM tris(hydroxymethyl)aminomethane-HCl, pH 7.4, 4 mM $MgCl_2$, 0.67 mM ATP, 0.67 mM cAMP, 0.1 mM GTP, 1 mM dithiothreitol, 1 mM ethyleneglycol bis(3-aminoethyl ether)-N,N,N′N′-tetraacetic acid, 5 mM creatine phosphate, 11 U of creatine phosphokinase, 1 mg/ml bovine serum albumin, 5 mM theophylline, 1 to 1.2 μCi of [α-^{32}P]ATP and 20 to 40 μg of membrane protein in a final volume of 150 μl. After the incubation for 15 min at 30 C, the reaction was terminated by adding 100 μl of 4 mM cAMP and boiling the samples for 5 min. The centrifuged samples were diluted by 1 ml of distilled water, and [^{3}H]cAMP (approximately 20,000 cpm) was added to monitor the chromatographic recovery of cAMP. cAMP was separated from ATP using sequential chromatography on Dowex and alumina columns as described by Salomon *et al.* (1974).

Protein assay. The protein content in membrane fractions was determined according to Lowry *et al.* (1951).

Data analysis. The data from individual binding experiments were analyzed by the IBM-compatible computer program EBDA (Dr. G. A. McPherson, Vanderbilt University; saturation studies) or CDATA 87 (EMF Software, Knoxville, TN; competition studies). In competition studies, the comparison of data fit for one-site or two-site models was based on a statistical test of residuals. K_i values were calculated from IC_{50} values as previously described according to the equation $K_i = IC_{50}/(1 + [L]/K_d)$ (Cheng and Prusoff, 1973; Leader *et al.*, 1989). Unless stated otherwise, the data are expressed as mean ± S.D. obtained from the number of experiments indicated in the text.

Results

Saturation analysis of [^{3}H]NMS binding in the subcellular fractions from circular smooth muscle/deep muscular plexus. The results of the saturation analysis of [^{3}H]NMS binding to membrane fractions prepared from the homogenates of the circular smooth muscle/deep muscular plexus are illustrated in figure 1. In the purified synaptosomal fraction (fig. 1A), the half-maximal saturation of binding sites (K_d) obtained with [^{3}H]NMS was 2.7 ± 0.4 nM, with a B_{max} value of 195 ± 44 fmol/mg protein (n = 4). In the purified smooth muscle plasma membranes (fig. 1B), the K_d value of [^{3}H]NMS binding was 16 ± 2 nM and the B_{max} = 2088 ± 276 fmol/mg (n = 4). These data were indicative that two different populations of muscarinic receptors were present in the membrane fractions obtained from circular smooth muscle/deep muscular plexus: the prejunctional muscarinic receptors found in the purified synaptosomal fraction, and the postjunctional muscarinic receptors associated with the smooth muscle plasma mem-

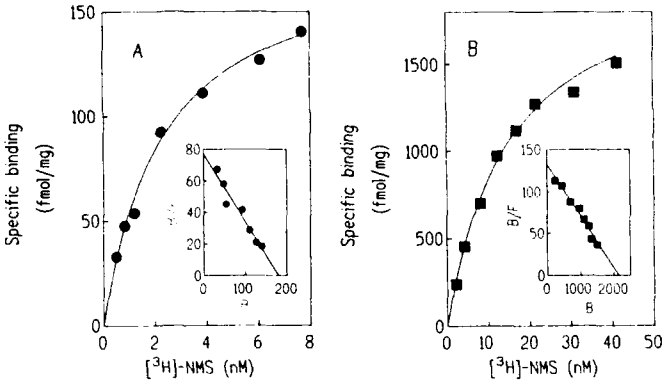

Fig. 1. Saturation analysis of [^{3}H]NMS binding to muscarinic receptors in the purified synaptosomal fraction (A) and in the smooth muscle plasma membranes (B) with the corresponding Scatchard plots (insets). The data points are means of triplicate measurements.

branes. Furthermore, these membrane populations can be distinguished based on their affinities for [^{3}H]NMS.

In either membrane fraction, the [^{3}H]NMS interacted with an apparently uniform population of binding sites because the Scatchard plots were linear (fig. 1, insets), and the Hill coefficients were unity ($n_H = 1.00 \pm 0.02$ in the purified synaptosomal fraction; 1.00 ± 0.03 in the purified smooth muscle plasma membranes; $n = 4$). These findings suggested that the membrane fractions contained only marginal amounts of cross-contamination.

Competition studies. To characterize the pharmacological properties of prejunctional and postjunctional muscarinic receptors in the circular smooth muscle/deep muscular plexus, subtype-selective muscarinic antagonists were used in [^{3}H]-NMS competition binding experiments. The results are summarized in figure 2 and table 1. The potencies of muscarinic antagonists on prejunctional receptors (fig. 2A) showed the following order: 4-DAMP > pirenzepine = methoctramine > AF-DX 116 (K_i values of 0.039, 2.54, 2.25, 6.20 μM, respectively). Each antagonist inhibited [^{3}H]NMS binding competitively because the Hill coefficients were close to unity.

A similar ranking of potencies of the four subtype-specific muscarinic antagonists was observed in competition studies with postjunctional receptors (fig. 2B). However, the affinities of 4-DAMP and pirenzepine were approximately 4- to 5-fold less potent compared to the corresponding affinities obtained with the prejunctional receptors. Conversely, the cardioselective compounds AF-DX 116 and methoctramine had comparable affinities for both prejunctional and postjunctional receptors. The inhibition of [^{3}H]NMS binding by AF-DX 116 to postjunctional receptors resulted in shallow competition curves with a Hill coefficient significantly less than 1.

Interaction of prejunctional receptors with adenylate cyclase. The primary transducing event triggered by the activation of postjunctional muscarinic receptors in the smooth muscles of gastrointestinal tract is the activation of phospholipase C, followed by the enhanced turnover of phosphoinositides and Ca^{++} mobilization (Ek and Nahorski, 1988; Salmon and Bolton, 1988). However, little is known about the signaling pathway(s) linked to the enteric prejunctional receptors. Thus, we next examined the activity of synaptosomal adenylate cyclase in response to the activation of prejunctional muscarinic receptors.

The basal adenylate cyclase activity in the crude synaptosomal fraction (689 ± 50 pmol of cAMP per milligram of protein per 15 minutes; $n = 7$) was stimulated approximately 3- to 4-fold in the presence of 10 μM forskolin. The muscarinic agonists carbachol and oxotremorine caused a concentration-dependent inhibition of forskolin-stimulated adenylate cyclase activity, and this inhibition was abolished in the presence of 1 μM atropine (fig. 3). These observations implicated the inhibition of adenylate cyclase activity in the mechanism(s) of signal transduction activated by the prejunctional muscarinic receptor in the deep muscular plexus. In view of studies demonstrating the involvement of phospholipase C in the propagation of signal from the gastrointestinal postjunctional receptors, the muscarinic receptor-mediated inhibition of adenylate cyclase in the present study showed fundamental differences in the mode of coupling of prejunctional and postjunctional receptors to the second messenger systems. However, involvement of phosphoinositide metabolites in the transducing pathway is possible, inasmuch as muscarinic receptors linked to the inhibition of adenylate cyclase were shown to accelerate the hydrolysis of phoshoinositides under certain circumstances (Bonner, 1989).

Discussion

To our knowledge, this study was the first to demonstrate the presence of prejunctional muscarinic receptors in the axonal varicosities of deep muscular plexus. These receptors could be identified based on their affinity for [^{3}H]NMS, and they exhibited several additional pharmacological differences compared to the postjunctional receptors of the circular smooth muscle. The evidence for the presence of both prejunctional and postjunctional muscarinic receptors in the circular smooth muscle/deep muscular plexus of canine ileum was derived from the

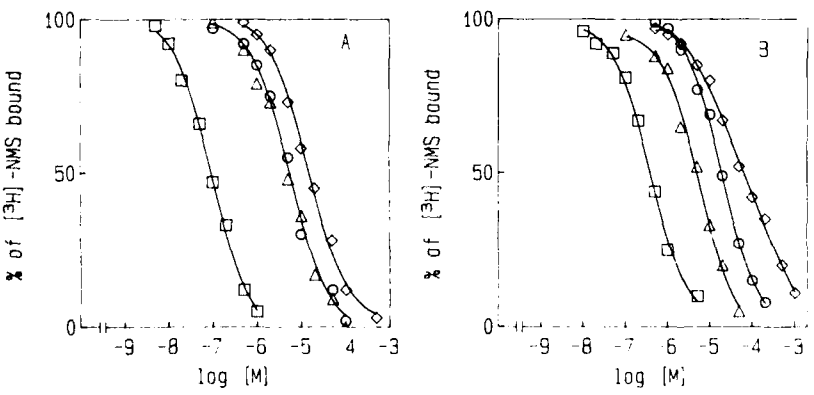

Fig. 2. Inhibition of [^{3}H]NMS binding by muscarinic antagonists 4-DAMP (squares), methoctramine (triangles), pirenzepine (circles) and AF-DX 116 (diamonds) in the purified synaptosomal fraction (A) and purified smooth muscle plasma membranes (B). The antagonist effects were measured simultaneously on the membrane fractions prepared from the same starting material. The ligand concentrations were near or greater than the K_d values (2.5–4 nM for presynaptic receptors; 15–20 nM for postjunctional receptors). The results represent means of triplicates from a single representative experiment with each antagonist. The average binding affinities, corrected for ligand occupancy (K_i values), were calculated from three independent experiments and are presented in table 1.

TABLE 1

Inhibition constants of subtype-specific muscarinic antagonists

Results are means ± S.D. of three experiments.

Antagonist	K_i	
	Prejunctional receptors	Postjunctional receptors
	μM (n_H)	
4-DAMP	0.039 ± 0.004	0.19 ± 0.02***
	(1.02 ± 0.02)	(1.00 ± 0.03)
Pirenzepine	2.54 ± 0.27	9.50 ± 0.42***
	(1.05 ± 0.04)	(1.01 ± 0.05)
Methoctramine	2.25 ± 0.29	2.21 ± 0.15
	(0.99 ± 0.03)	(0.98 ± 0.03)
AF-DX 116	6.20 ± 0.27	12.5 ± 0.6***[a]
	(0.97 ± 0.04)	(0.71 ± 0.05**)

[a] Refers to the apparent K_i value ($n_H < 1$).

** Significantly different from unity ($P < .01$); *** significantly different from the corresponding K_i value for prejunctional receptors ($P < .001$).

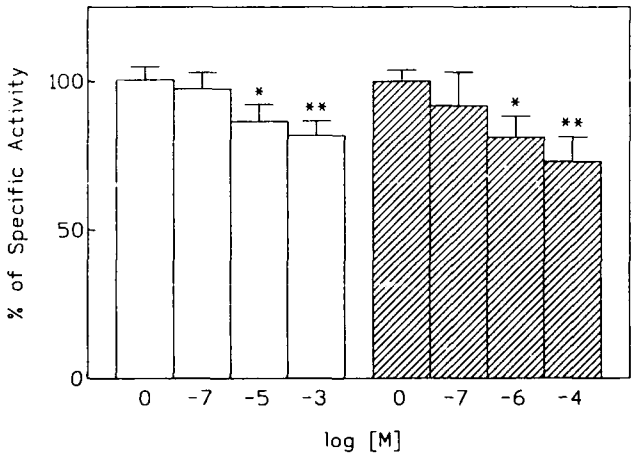

Fig. 3. Muscarinic receptor-mediated inhibition of forskolin-stimulated adenylate cyclase activity in the crude synaptosomal fraction from the circular smooth muscle/deep muscular plexus. The samples, containing 20 to 40 μg of membrane protein, were incubated in the presence of 10 μM forskolin with either carbachol (open bars) or oxotremorine (diagonal hatched bars) at molar concentrations indicated under the abscissa. The adenylate cyclase activity is expressed as percent of forskolin-stimulated response. The results are mean values from triplicate measurements of adenylate cyclase activity on four membrane preparations. The standard deviations are indicated by vertical lines at the top of the bars. The data were analyzed by one-way analysis of variance and the statistical significance of differences in between-column means was determined by Scheffe's test. In the presence of 1 μM atropine, the adenylate cyclase activity observed with 0.1 mM oxotremorine (103 ± 6%) or 1 mM carbachol (101 ± 5%) was not significantly different from the activity in the presence of forskolin alone ($P > .2$). *$P < .05$; **$P < .01$.

examination of binding properties of these receptors in the purified synaptosomal fraction and in purified smooth muscle plasma membranes. The association of synaptosomal binding activity with axonal varicosities of deep muscular plexus was supported by several observations. First, it has been shown that the purified synaptosomal fraction is selectively enriched in neuron-specific markers such as [^{3}H]saxitoxin binding and the amount of immunoreactive vasoactive intestinal peptide (Ahmad *et al.*, 1988; Gordon *et al.*, 1990). Furthermore, the synaptosomal fraction, unlike smooth muscle plasma membranes, was found to contain a high level of opioid receptors, reflecting their predominant, if not exclusive, association with the enteric nerves (Allescher *et al.*, 1989). In addition, the anatomical organization of the deep muscular plexus, *i.e.*, the absence of neuronal cell bodies, precludes the assignment of the synaptosomal binding activity to plasma membranes of neuronal cell bodies. Lastly, significant differences in the muscarinic receptor binding between the synaptosomal fraction and the smooth muscle plasma membrane fraction obviate the concern that the synaptosomal binding activity may be derived from the contaminating smooth muscle plasmalemma.

In examining the pharmacological properties of prejunctional receptors, we found that the M_3/M_1 subtype-selective antagonist 4-DAMP was the most potent inhibitor of [^{3}H]NMS binding in the synaptosomal fraction. The high affinity of 4-DAMP for gastrointestinal prejunctional receptors has also been observed in electrophysiological and binding studies using myenteric and submucosal plexuses (North *et al.*, 1985; Kostka *et al.*, 1989a). On the other hand, the M_1-selective antagonist pirenzepine and the cardioselective compounds methoctramine and AF-DX 116 interacted with low affinity at the prejunctional receptors in the present study. Thus, the prejunctional muscarinic receptors in the ileum might have a pharmacological profile of the M_3 subtype. However, this was at variance with the present and past observations of muscarinic receptor-mediated inhibition of adenylate cyclase in the synaptosomal fractions from myenteric and deep muscular plexuses (Kostka *et al.*, 1989b). Also, others have shown that the M_3 subtype mediates primarily the excitatory responses, using the enhanced turnover of phosphoinositides and Ca^{++} mobilization as a transducing pathway (Noronha-Blob *et al.*, 1987; Jacobson *et al.*, 1985).

Prejunctional receptors were shown to negatively modulate adenylate cyclase activity, and recent cloning studies have identified two different subtypes of muscarinic receptors, m2 and m4 (Bonner *et al.*, 1987; Peralta *et al.*, 1987), which were also linked to the inhibition of adenylate cyclase. The m2 receptors resemble the pharmacologically defined M_2 (cardiac) receptors. Inasmuch as the binding data from the prejunctional receptors in deep muscular plexus (particularly their low affinities for cardioselective antagonists methoctramine and AF-DX 116) clearly showed a lack of similarity to cardiac M_2 receptors, the prejunctional receptors studied here might resemble the cloned m4 receptor. This conclusion was recently proposed by Ehlert *et al.* (1989) to account for the pharmacological properties of adenylate cyclase-linked muscarinic receptors in the rat striatum. The striatal receptors were found to have a high affinity for 4-DAMP and low affinities for pirenzepine, gallamine and AF-DX 116. The m4 receptor subtype has been shown to have low affinity for pirenzepine and cardioselective agents (Buckley *et al.*, 1989).

The functional role of prejunctional muscarinic receptors in the deep muscular plexus remains to be elucidated. Most likely, these receptors will have a function similar to that of the prejunctional receptors of myenteric and submucosal plexuses, that is, the negative control of neurotransmitter release (Briggs and Cooper, 1982; North *et al.*, 1985).

The properties of postjunctional muscarinic receptors associated with the circular smooth muscle were studied by examining the characteristics of [^{3}H]NMS binding to the fraction of purified smooth muscle plasma membranes. In competition studies, none of the tested antagonists exhibited potent inhibition of [^{3}H]NMS binding. The low potencies of pirenzepine, AF-DX 116 and methoctramine suggested an absence of either M_1 or M_2 receptor subtypes in canine circular muscle. However, the relatively low potency of 4-DAMP precluded the classification of these receptors as being of M_3 category.

Previous binding studies have suggested that the postjunctional receptors on gastrointestinal smooth muscles exhibit the properties of a mixture of M_2 and M_3 receptors, *i.e.*, some subtype-selective muscarinic antagonists such as AF-DX 116, methoctramine and hexahydrosiladifenidol display a complex behavior in competition curves, which could be resolved into two independent sites (Giraldo *et al.*, 1987, 1988; Michel and Whiting, 1988; Gordon *et al.*, 1989). In the present study, the competition experiments did not reveal heterogeneity of binding sites in the smooth muscle plasma membrane fraction. Except for AF-DX 116, the Hill coefficients for the four subtype-specific muscarinic antagonists were not significantly different from unity. Although the competition experiments with AF-DX 116 resulted in shallow curves and Hill coefficients significantly less than 1, the computer-assisted resolution of binding data into a two-site model did not improve the fit. Considering the relatively low potency of AF-DX 116 in the present model (apparent K_i value of 1.2 μM), it was likely that the complex behavior of this compound might be related to the presence of allosteric effects. Similarly, allosteric effects of AF-DX 116 have been reported for smooth muscle muscarinic receptors (Roffel *et al.*, 1989). Therefore, the binding data in the present study did not permit the clear-cut delineation of a pharmacological subtype(s) for the postjunctional muscarinic receptor in the circular smooth muscle. However, it could not be excluded that the muscarinic receptor(s) in the gastrointestinal tract might represent a category that is distinct from the three major pharmacological classes of muscarinic receptors (Batink *et al.*, 1987; Bonner *et al.*, 1987; Buckley *et al.*, 1989; Kostka *et al.*, 1989a; Peralta *et al.*, 1987).

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